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The allantois and chorion, when isolated before circulation or chorio-allantoic fusion, have hematopoietic potential

Brandon M. Zeigler¹, Daisuke Sugiyama^{1,*}, Michael Chen¹, Yalin Guo¹, Karen M. Downs^{2,†} and Nancy A. Speck^{1,†}

The chorio-allantoic placenta forms through the fusion of the allantois (progenitor tissue of the umbilical cord), with the chorionic plate. The murine placenta contains high levels of hematopoietic stem cells, and is therefore a stem cell niche. However, it is not known whether the placenta is a site of hematopoietic cell emergence, or whether hematopoietic cells originate from other sites in the conceptus and then colonize the placenta. Here, we show that the allantois and chorion, isolated prior to the establishment of circulation, have the potential to give rise to myeloid and definitive erythroid cells following explant culture. We further show that the hematopoietic potential of the allantois and chorion does not require their union, indicating that it is an intrinsic property of these tissues. These results suggest that the placenta is not only a niche for, but also a source of, hematopoietic cells.

KEY WORDS: Placenta, Allantois, Chorion, Hematopoiesis, Mouse

INTRODUCTION

Hematopoietic cells develop from mesoderm located in multiple sites in the conceptus, beginning in the mouse at the mid-primitive-streak stage [~7.0 days postcoitum (dpc)] (Palis et al., 1999). The sites of hematopoietic cell emergence in mammals have been defined using hematopoietic progenitor assays or, in the case of hematopoietic stem cells, by transplantation into newborn, irradiated, or otherwise compromised adult mice (for reviews, see Dzierzak, 2005; Speck et al., 2002). Sites defined by these approaches include: the yolk sac, which gives rise to both primitive and definitive blood cells; the aorta/gonad/mesonephros region and its precursor, the para-aortic splanchnopleura (PAS); and the umbilical artery (Cumano et al., 1996; de Bruijn et al., 2000; Medvinsky and Dzierzak, 1996; Moore and Metcalf, 1970; Müller et al., 1994; Palis et al., 1999; Weissman et al., 1978; Yoder et al., 1997).

In addition to sites of hematopoietic cell emergence, two hematopoietic cell niches have been identified within the liver and placenta in the mid-gestation mouse conceptus (~10.0–11.0 dpc). It has long been accepted that the fetal liver is a site of hematopoietic colonization and is not an intrinsic source of hematopoietic cells (Houssaint, 1981). However, it is not known whether the placenta, whose contribution to mammalian fetal hematopoiesis was more recently appreciated (Alvarez-Silva et al., 2003; Gekas et al., 2005; Melchers, 1979; Ottersbach and Dzierzak, 2005) is simply a site of colonization (like the fetal liver), or whether it is also a source of hematopoietic cells. Determining whether any tissue is truly a site of hematopoietic cell emergence is complicated by the fact that hematopoietic cells circulate throughout the conceptus. Thus, in this study, we have investigated the hematopoietic potential of the two

individual components of the murine placenta, the allantois and the chorion, prior to establishment of vascular continuity within the conceptus.

The chorion arises during gastrulation (~7.0–7.25 dpc) by displacement of extra-embryonic ectoderm onto the roof of the newly formed exocoelomic cavity (Snell and Stevens, 1966). It is a bilayered tissue of dual origin, composed of extra-embryonic ectoderm derived from trophoblast and extra-embryonic mesoderm derived from proximal epiblast (Gardner, 1983; Kinder et al., 1999; Lawson et al., 1991) (Fig. 1A). The ectodermal component of the chorion differentiates into syncytio- and cytotrophoblasts, which mediate fetal/maternal exchange.

The murine allantois is thought to be composed wholly of extra-embryonic mesoderm (Duval, 1891) derived from proximal epiblast that is transformed into mesoderm via passage through the posterior primitive streak (Beddington, 1982; Copp et al., 1986; Kinder et al., 1999; Lawson et al., 1991; Tam and Beddington, 1987). The allantois appears slightly later than the chorion during gastrulation, and emerges first as a bud emanating from the posterior region of the streak (~7.25 dpc). The bud enlarges within the exocoelomic cavity by a combination of mitosis, continued cellular addition from the primitive streak and distal cavitation (Brown and Papaioannou, 1993; Downs and Bertler, 2000; Ellington, 1985; Tam and Beddington, 1987). Ultimately, the allantois elongates far enough to make contact with the chorionic mesoderm, and fuses with it [6–8 somite pairs (s), ~8.5 dpc]. Chorio-allantoic union is mediated by the maturity of the allantois (Downs and Gardner, 1995), and requires both the cell-surface molecule vascular cell adhesion molecule 1 (VCAM1) on the outer mesothelial surface of the allantois (Gurtner et al., 1995; Kwee et al., 1995) and its counter receptor $\alpha 4$ integrin, on chorionic mesoderm (Yang et al., 1995). Together the allantois and chorion give rise to the labyrinth region of the placenta.

Shortly after the allantoic bud is formed, allantoic mesoderm vascularizes *de novo* (~7.5 dpc). Vascularization occurs with distal-to-proximal polarity, spreading toward the posterior end of the primitive streak (Downs et al., 1998). At about the same time, vasculogenesis takes place in the yolk sac blood islands and heart region, and spreads toward the posterior streak. By 4–6 s, the three major circulatory systems unite near the base of the allantois,

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forming a vascular continuum throughout the conceptus (Downs, 1998; Inman and Downs, 2006). By 8 s (~8.5–8.75 dpc), primitive erythroid cells can be seen freely circulating within the allantois (Downs et al., 1998).

Although erythroid cells could be detected in the murine allantois in early somite pair conceptuses, it was not entirely clear whether or not they originated from the allantois. In avian embryos, the apparently prevascularized allantois appears to be a hematopoietic tissue and contains blood-island-like clusters of hematopoietic cells (Caprioli et al., 1998; Caprioli et al., 2001). Dieterlen-Lièvre and colleagues also showed that the avian allantois could contribute extensively to hematopoietic and endothelial cells in the adult bone marrow following engraftment into the coelom of host embryos, and thus has the potential to form definitive (adult) blood (Caprioli et al., 1998). In contrast, when headfold-stage murine allantoises were labeled intrinsically with lacZ and extrinsically with tritiated thymidine, and then grafted into the exocoelomic cavity of unlabeled hosts to form chimeric allantoises, no triply labeled (β -galactosidase⁺/tritium⁺/benzidine⁺) donor-derived erythroid cells were detected in the host allantoises following 24 hours of whole embryo culture (Downs et al., 1998). Furthermore, those explants that were free-floating in the exocoelom or fused only with the chorion and did not exhibit vascular continuity with the fetus and/or yolk sac were entirely devoid of benzidine-positive cells, suggesting that, in the mouse conceptus, the allantois is not a source of erythroid cells (Downs et al., 1998). However, some data suggested that the murine allantois might have erythropoietic potential: 5–10% of allantoises explanted between the neural plate and 4 s stages (~7.5–8.25 dpc), or cultured in isolation for 24 hours exhibited a constant but relatively small population of erythroid cells, the origin of which has never been accounted for (Downs et al., 1998).

The aforementioned studies of the murine allantois were carried out during only a short period (24 hours), and early markers of hematopoietic cells were not examined. In light of recent experiments suggesting that hematopoietic cells emerge from the placenta several days later (Alvarez-Silva et al., 2003; Gekas et al., 2005; Ottersbach and Dzierzak, 2005), we undertook a series of experiments to examine the hematopoietic potential of the allantois and the chorion prior to their fusion and, more importantly, prior to the vascular link-up between the umbilical, yolk sac and cardiovascular systems in the conceptus. First, we followed the expression of *Runx1* in both the intact conceptus and in explant cultures. *Runx1* is a transcription factor required for hematopoietic stem cell formation, and is also an early marker for sites of hematopoietic cell emergence in the embryo (Cai et al., 2000; Cia-Uitz et al., 2000; Kalev-Zhylynska et al., 2002; Lacaud et al., 2002; North et al., 2002; North et al., 1999; Okuda et al., 1996; Wang et al., 1996). *Runx1* expression has been documented in the placenta (Ottersbach and Dzierzak, 2005), at the site of chorio-allantoic fusion (North et al., 1999) and in the chorion prior to fusion (Lacaud et al., 2002), but has not been reported in the pre-fusion allantois. Second, we cultured allantoises and chorions on OP9 stromal cells in the presence of hematopoietic cytokines, or as explants on plastic followed by methylcellulose colony forming assays, to determine their hematopoietic potential.

Here we show that both the allantois and chorion, having been isolated from early headfold-stage conceptuses before establishment of the circulation, express *Runx1* and exhibit hematopoietic potential following explant culture. Furthermore, we show that this hematopoietic potential is not dependent on chorio-allantoic fusion, indicating that it is an intrinsic property of both tissues.

MATERIALS AND METHODS

Mouse husbandry

All mice were maintained in light-reversed conditions (lights off: 12:00/ lights on: 0:00). *Runx1*-deficient explants were generated by mating *Runx1*^{td/+} females (Wang et al., 1996) with *Runx1*^{l^z/+} males (North et al., 1999) (formal designations of the *Runx1*^{td} and *Runx1*^{l^z} alleles are *Runx1*^{tm1Spe} and *Runx1*^{tm2Spe}, respectively). For marking experiments, B6.Cg-Tg(ACTB-Bgeo/GFP)21Lbe/J (Jackson Laboratory, Bar Harbor, ME), *Tg(Ly6a/GFP)* (Ottersbach and Dzierzak, 2005), or *Runx1*^{l^z/+} male mice were bred to female C57BL/6J, C57BL/6J × 129S1/SVImJ F1, or B6CBAF1/J mice (Jackson Laboratory). We identified female mice in estrus, mated them 5 minutes before the beginning of the dark cycle and then examined them 4 hours later for the presence of a vaginal plug. Dissections, staging and morphological scoring were performed as previously described (Downs, 2006; Downs and Davies, 1993; Downs and Harmann, 1997; Downs et al., 2004). All mouse procedures were approved by our Institutions' Animal Care and Use Committees.

Embryo dissection

We isolated allantoises by mouth aspiration (Downs, 2006) (Fig. 2A), and in some cases subdivided the allantoises into distal, mid and proximal thirds with glass scalpels.

To isolate the chorion, the Reichert's membrane was removed and the embryonic and visceral endoderm was labeled by submerging the intact conceptus for 1 minute in AlexaFluor594-conjugated Concanavalin A (ConA 594) [5 mg/ml in phosphate-buffered saline (PBS)] (Molecular Probes/Invitrogen, Carlsbad, CA) (Fig. 2B). The mesoderm lining the exocoelomic cavity was labeled by injecting AlexaFluor488-conjugated Concanavalin A (ConA 488) into the exocoelomic cavity using a mouth-held microcapillary pipette. Tissues were digested using pancreatin/trypsin (Downs, 2006) for 10 minutes at 4°C and separated with the aid of glass scalpels and a mouth aspirator. Separation of the chorion from visceral endoderm was confirmed by the absence of ConA 594 and the presence of ConA 488 by fluorescence stereomicroscopy (Leica, Ernst-Leitz-Strasse, Germany). Once this technique was established, we labeled only the endoderm by dipping the conceptus into ConA 488 and removing the ConA 488-positive tissue from the chorion.

We isolated PAS as described by Godin et al. (Godin et al., 1993). We isolated embryonic ectoderm by incubating headfold-stage conceptuses in ConA 488 to label the anterior head endoderm (Fig. 2C). A transverse cut was made below the amnion using a glass scalpel to separate the extra-embryonic from embryonic components of the conceptus. A sagittal cut was then made through the epiblast; the anterior portion of the epiblast was saved. The tissues were digested in trypsin/pancreatin at 4°C for 10 minutes and then dissected using a glass needle and mouth aspirator to separate the anterior neuro-ectoderm and mesoderm from the anterior head endoderm.

Explant cultures for *Runx1* and *Ly6A* expression

Allantoises and chorions were cultured as explants directly on plastic (24 Well Cell Culture Cluster, Corning, Corning, NY) or in roller drum suspensions for 48 hours (Downs, 2006). Most material was at headfold stage, with a minor 1–2 s component. We fixed the explants in 4% paraformaldehyde for 2 hours on ice; they were then washed with PBS and stained for 6 hours for β -galactosidase activity (Miles et al., 1997). The genotype of explants from *Runx1*^{l^z/+} × *Runx1*^{td/+} matings was determined by PCR from corresponding epiblast DNA using previously described primers and amplification conditions (North et al., 1999).

OP9 explant cultures

Individual allantois, chorion, PAS, visceral endoderm and neuroectoderm explants were added to a freshly prepared layer of OP9 stromal cells in single wells of 12-well plates and grown in 1 ml α -MEM (Gibco/Invitrogen) containing 20% fetal bovine serum supplemented with stem cell factor (SCF, 50 ng/ml), interleukin 3 (IL3, 5 ng/ml), interleukin 6 (IL6, 5 ng/ml), Flt-3 ligand (Flt3-L, 10 ng/ml), granulocyte colony stimulating factor (G-CSF, 5 ng/ml), granulocyte monocyte stimulating factor (GM-CSF, 5 ng/ml) (all cytokines from R&D Systems, Minneapolis, MN) and 2-mercaptoethanol (2-ME, 1×10^{-5} M) (Sigma) at 37°C in 5% CO₂ (Nishikawa et al., 1998) to

encourage the proliferation and differentiation of myeloid lineage cells. Half of the medium and cytokines were replaced every other day. Explant cultures were analyzed for DiI-Ac-LDL (Biomedical Technologies, Stoughton, MA) uptake or CD45 expression after 7 days of culture. Fluorescence activated cell sorting (FACS) analyses were performed after 14 days of explant culture.

Flow cytometry

Single-cell suspensions were prepared by incubating the explants for 10 minutes in 500 μ l of Cell Dissociation Buffer (Gibco/Invitrogen) at 37°C followed by passage through a 40- μ m strainer. Explant cell suspensions at a concentration of 2×10^6 cells per ml were treated with Fc blocking serum (BD Pharmingen, San Diego, CA) for 20 minutes at 4°C then incubated with CD45-PerCP (30-F11), Mac1-PE (M11/70), c-kit-FITC (2B8), or Gr-1-FITC (RB6-8SC) (BD Pharmingen or eBioscience, San Diego, CA), washed and re-suspended in 200 μ l FACS buffer (BioSure, Grass Valley, CA). Dead cells were excluded with TO-PRO-3 (Molecular Probes/Invitrogen), and cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Rockville, MD) with FloJo software (Tree Star Inc., Ashland, OR).

Progenitor assays

Two-day explant cultures were performed by plating six conceptus equivalents of allantoises or chorions in a single well of a 24-well plate containing 500 μ L of 50% rat serum/DMEM (Downs et al., 2001). The medium was replaced after 24 hours and at 48 hours the explants were washed in PBS, then incubated in pre-heated (37°C) Dispase II (Roche, Basel, Switzerland) for 25 minutes and mechanically dissociated using a pipette. Cells were collected by centrifugation and cultured in 3.3 ml of 3434 Methocult (StemCell Technologies, Vancouver, Canada). Colonies were counted and selected 7 days later.

Five-day explant cultures were performed by plating six conceptus equivalents of allantoises or chorions on OP9 cells in a single well of a 24-well plate in the presence of 50 ng/ml SCF, 10 ng/ml Flt3-L, 4 U/ml erythropoietin (EPO) and 1×10^{-5} M 2-ME to preferentially encourage the proliferation of erythroid progenitors. Explants were dissociated into single-cell suspensions as described above and plated in 3.3 ml of 3434 MethoCult (Stem Cell Technologies). Colonies were scored and harvested 10 days later.

β -Globin expression analysis

RNA was extracted from individual burst-forming unit erythroid (BFU-E) or colony-forming unit granulocyte-macrophage (CFU-GM) colonies, adult mouse bone marrow, and ~9.0 dpc mouse yolk sac using an RNA extraction kit (Qiagen, Valencia, CA). ϵ -Globin (ϵ y) primers (5' TGT CCT CTG CCT CTG CCA TAA 3' and 5' AGC GGA CAC ACA GGA TTG CTG 3') (Fiering et al., 1995), β -major globin primers (5'-CTG ACA GAT GCT CTC TTG GG-3' and 5'-CAC AAA CCC CAG AAA CAG ACA-3') and *Hprt* primers (5'GCT GGT GAA AAG GAC CTC T-3' and 5'-CAC AGG ACT AGA ACA CCT GC-3') were used to analyze globin expression (Keller et al., 1993). PCR was performed for 29 cycles: 94°C, 30 seconds; 57°C, 30 seconds; 72°C, 45 seconds.

Histological analysis

Conceptuses were assayed for β -galactosidase activity (Miles et al., 1997), and 5 μ m paraffin sections were either counterstained with Nuclear Fast Red (Vector Labs, Burlingame, CA) or left unstained. Staining for β -galactosidase was performed for 6 hours at 37°C, unless otherwise noted in the figure legends. We examined five conceptuses at bud stages, eight at headfold stages, 17 at 1-16 s and two at 9.5 dpc.

Explants from OP9 cultures were incubated in 1 μ l/ml DiI-Ac-LDL for 4 hours at 37°C, fixed in 4% paraformaldehyde, and then incubated first with CD45-biotinylated antibody (BD Pharmingen) overnight at 4°C and then with Pacific Blue-conjugated streptavidin (Molecular Probes/Invitrogen) for 90 minutes at room temperature. Explants were observed by fluorescence stereomicroscopy and photographed using a monochrome digital camera (Roper Scientific, Tucson, AZ).

Single-cell suspensions of explants (1×10^4 cells) or individually selected colonies were centrifuged onto slides (14 g, 8 minutes), dried for 3 hours at room temperature and stained by May-Grunwald Giemsa.

Cytospin preparations were observed by compound microscopy and photographed using a digital color camera (Diagnostic Instruments, Sterling Heights, MI).

All explant experiments were performed at least three times on headfold-stage material.

RESULTS

Runx1 is expressed in the allantois and the chorion

We examined the expression of Runx1 in the intact conceptus prior to and shortly after chorio-allantoic fusion (~7.5-9.25 dpc) (Fig. 1). We and others previously reported that at stages prior to fusion, Runx1 is expressed in yolk sac visceral endoderm (North et al., 1999), the vascular and hematopoietic components of yolk sac blood islands (Lacaud et al., 2002; North et al., 1999), and in chorionic mesoderm (Lacaud et al., 2002). We observed Runx1 expression, as visualized from a lacZ 'knock-in' allele (North et al., 1999), in these same locations (Fig. 1B). Runx1 expression in the chorion was first seen as punctate blue staining in some mesodermal cells beginning at the early bud stage (not shown), and was intense in most chorionic mesodermal cells by the late headfold stage (Fig. 1B). Chorionic ectoderm was negative at all stages examined. In the allantois, we found convincing blue stain within the proximal allantois and amnion where these two tissues join, as well as faint blue stain within the distal portion of the allantois, including the outer layer of mesothelium in some specimens (not shown). After chorio-allantoic fusion (12 s, ~9.0 dpc), Runx1 expression was seen within internal cells at the chorio-allantoic interface (Fig. 1C-E), at external sites where the allantois intersects the chorion (Fig. 1E, blue arrows) and also in a small cluster of cells within the base of the allantois (Fig. 1E,F). At later times (~16 s, ~9.25 dpc), Runx1 expression was seen both at the plane of chorio-allantoic fusion (Fig. 1G) and in endothelial cells lining the proximal allantoic vasculature (Fig. 1G,H) but not in the mid-region of the allantois (Fig. 1G). Runx1 expression in allantoic mesothelium was clearly seen in the 9.5 dpc conceptus (Fig. 1I,J). By ~10.5 dpc, the umbilical artery, which forms from the allantois, expressed Runx1 throughout its length both in endothelial cells and in intra-aortic hematopoietic clusters (Fig. 1K,L) as previously described (North et al., 1999). In previous studies with the same *Runx1^{lacZ}* allele, Ottersbach and Dzierzak (Ottersbach and Dzierzak, 2005) showed that by ~11.0 dpc, Runx1 is expressed in the labyrinth region of the placenta in endothelial cells, in cells underlying the endothelium and in cells within the circulation.

Faint, punctate blue staining was observed in both endodermal and mesodermal components of the visceral yolk sac as previously described (North et al., 1999) (not shown), and in extra-embryonic visceral endoderm overlying the chorionic ectoderm ['distal' extra-embryonic endoderm or 'ectoplacental' endoderm (Duval, 1891)] (Fig. 1B).

Runx1 is expressed in both the allantois and the chorion, independent of their union

Although our results of histology clearly revealed Runx1 expression in the chorion prior to chorio-allantoic fusion, allantoic mesothelial expression was much less intense. In addition, once chorio-allantoic fusion has occurred it is no longer possible to determine whether the Runx1⁺ cells at the fusion junction originated from the allantois, from the chorion, or from both tissues. Therefore, to confirm that both the allantois and chorion express Runx1, we isolated both tissues prior to their fusion, cultured them in vitro as explants and analyzed the explants for Runx1 expression.

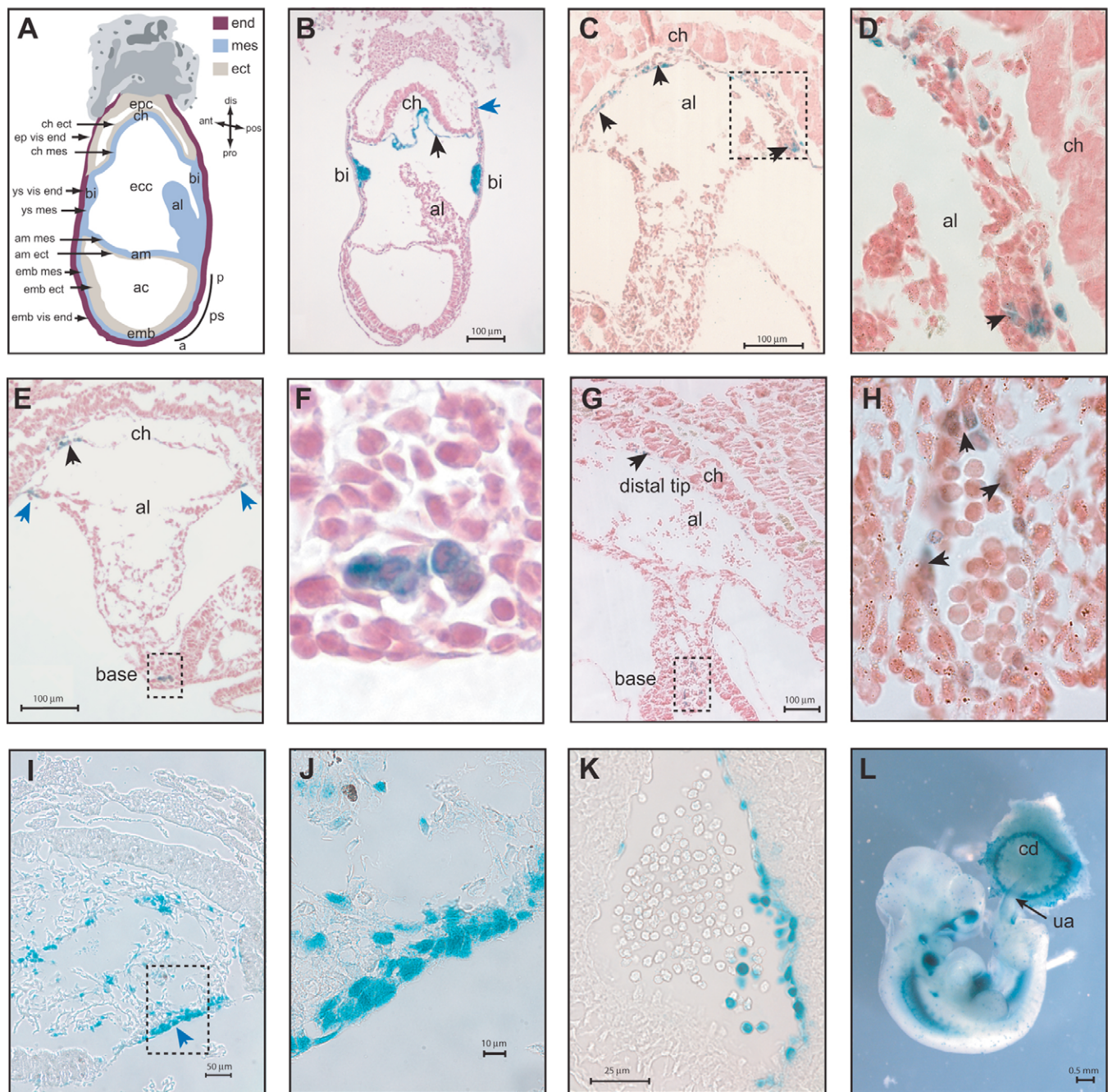


Fig. 1. Runx1 expression before and after chorio-allantoic fusion. (A) Schematic diagram of an early headfold (EHF) stage mouse embryo, showing locations of the ectoplacental cavity (epc), chorion (ch), exocoelomic cavity (ecc), amnion (am), amniotic cavity (ac), embryo (emb), yolk sac blood islands (bi) and allantois (al). Shown in color and labeled are both embryonic and extra-embryonic endoderm layer (end), mesoderm layer (mes) and ectoderm layer (ect). The conceptus is surrounded by embryonic (emb), yolk sac (ys) and ectoplacental (ep) visceral endoderm (vis end). The chorion consists of chorionic mesoderm (ch mes) and ectoderm (ch ect), the latter of which is derived from trophoblast. The anterior (a) and posterior (p) boundaries of the primitive streak (ps) are indicated. Compass shows anterior (ant), posterior (pos), proximal (pro) and distal (dis) directions for the extra-embryonic compartment. (B) Sagittal section of an EHF-stage *Runx1*^{+/+} conceptus showing Runx1 expression (blue) in the chorionic (ch) mesoderm (black arrow) and blood islands (bi). The blue arrow indicates area of punctate blue staining in ectoplacental visceral endoderm. β -Galactosidase staining was performed for 18 hours. (C) Runx1 expression (arrowheads) at the chorio-allantoic fusion junction in a 12 s *Runx1*^{+/+} conceptus. (D) Detail of Runx1 expression from boxed region in C. (E) Runx1 expression in a 12 s *Runx1*^{+/+} conceptus at the plane of chorio-allantoic fusion (black arrow), at the junctions between the allantois and chorion (blue arrows) and in a cluster of cells within the base (boxed region). β -Galactosidase staining was performed for 18 hours. (F) Detail of Runx1 expression from boxed region in E. (G) Runx1 expression in the distal region (arrowhead) and within the allantoic base (box) in a 16 s *Runx1*^{+/+} conceptus. (H) Detail of Runx1 expression (boxed region in G) in putative endothelial cells (arrowheads) lining a blood vessel within the allantoic base. (I) Section through placenta of a 9.5 dpc *Runx1*^{+/+} embryo showing Runx1⁺ cells in allantoic mesothelium (arrow). (J) Detail of boxed region from I. (K) Runx1 expression in endothelium and umbilical artery hematopoietic clusters in a 10.5 dpc *Runx1*^{+/+} conceptus. (L) Whole mount showing Runx1 expression in the chorionic disk (cd) and along the length of the umbilical artery (ua) of a 10.5 dpc *Runx1*^{+/+} conceptus.

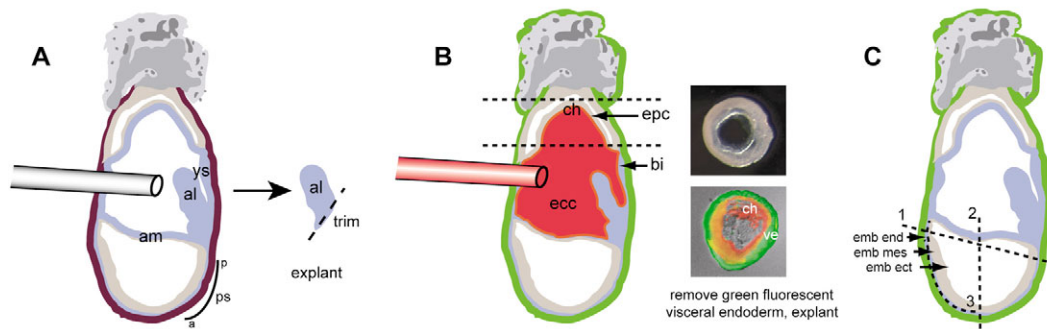


Fig. 2. Isolation of the allantois and chorion. (A) Schematic diagram illustrating isolation of the allantois (al) with a glass microcapillary pipette. Several cell layers were trimmed from the base with a glass scalpel. am, amnion; ys, yolk sac. (B) Chorion (ch) isolation. Endoderm was labeled by submerging the conceptus in ConA 594 (green) and injecting ConA 488 (red) into the exocoelomic cavity (ecc) to label the mesoderm that lines the cavity. bi, blood island; epc, ectoplacental cavity. Two incisions (dotted lines) were made to isolate the chorionic plate, which is shown in the right hand panels. We then trimmed visceral endoderm (ve, green) away from the chorion (ch, red) with a glass scalpel. After 24 hours of explant culture, chorions isolated in this way had no visible traces of green fluorescent ve-derived cells (Fig. 4A,B). (C) Isolation of anterior embryonic ectoderm (control for Table 1). The endoderm was labeled with ConA 594 (green). Three cuts were made with glass scalpels: 1, a transverse cut proximal to the amnion; 2, a sagittal cut to isolate the anterior portion of the embryo; 3, the embryonic endoderm and mesoderm were removed from the ectoderm with pancreatin/trypsin digest followed by excision with a glass scalpel.

As previously defined, the boundary of the allantois with the posterior primitive streak was taken as the site of insertion of the allantois with the amnion and yolk sac (Downs and Harmann, 1997). When aspirated via a mouth-held microcapillary pipette, the entire allantois can be removed from these insertion sites. We aspirated allantoises from early headfold to 1-2 s conceptuses with a glass pipette (Fig. 2A). Following aspiration, we carefully trimmed several cell layers from the base of the allantois with a glass scalpel to ensure that the allantoises were not contaminated with yolk-sac-derived cells. Allantoises from *Runx1*^{l2/+} conceptuses were then cultured for 48 hours on plastic, after which time the explants were assayed for β -galactosidase activity. We estimated that 48-hour explants were the equivalent of ~ 9.75 dpc (~ 20 s), as allantoises appear to have an internal timing mechanism revealed by their normal behavior even when removed from contact with the primitive streak (Downs and Gardner, 1995; Downs et al., 2004; Downs et al., 2001). Plated allantoic explants gave rise to *Runx1*⁺ cells (Fig. 3A).

Runx1⁺ cells included cells that remained within the explant, as well as mesenchymal-like cells that had crawled off the explant (Fig. 3A). These motile cells were previously shown by limited fate mapping to originate within the allantoic mesothelium (Downs et al., 2004), confirming that at least some of the *Runx1*⁺ cells came from the mesothelial component of the allantois.

To rule out the possibility that *Runx1* expression in the explants was induced nonspecifically through contact with the plastic surface of the culture well, we suspended explanted allantoises in roller tubes and cultured them in vitro for 48 hours (equivalent to ~ 9.75 dpc). *Runx1* expression was observed in the spheres that formed under these culture conditions (Fig. 3C). Sectioning through the spheres revealed that *Runx1* was expressed in the outer one to two cell layers around the surface of the spheres (Fig. 3C), consistent with expression in mesothelium. To discover which region of the allantois gave rise to *Runx1*⁺ cells, we also subdivided headfold-stage allantoises into distal, mid and proximal thirds and cultured

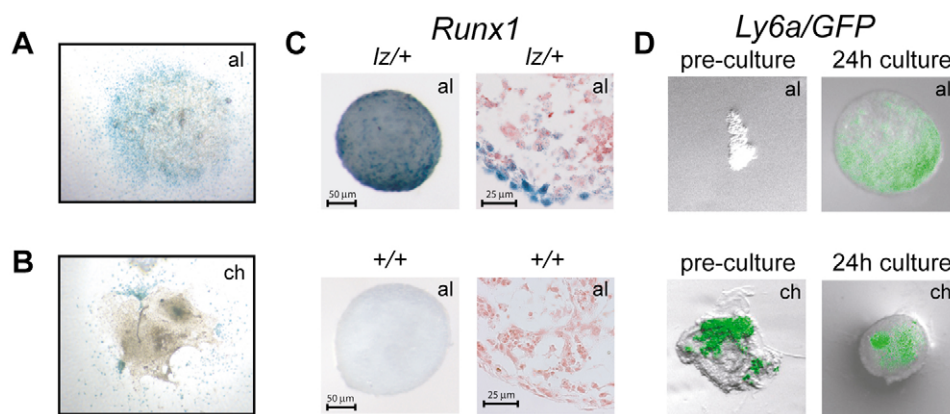


Fig. 3. *Runx1* expression in the allantois and chorion does not require chorio-allantoic fusion. (A) Allantois (al) explant culture on plastic (48 hours) from a *Runx1*^{l2/+} conceptus showing *Runx1* expression (blue) (3.2 \times). (B) Chorion (ch) explant culture on plastic from a *Runx1*^{l2/+} conceptus (3.2 \times). (C) Allantois sphere cultures (48 hours) from *Runx1*^{l2/+} (l2/+) and wild-type (+/+) conceptuses (left). Right hand panels are nuclear fast red stained histological sections of whole mount preparations of allantoic spheres showing *Runx1* expression predominantly in mesothelium on the surface of the sphere. (D) *Tg(Ly6a/GFP)* (Sca-1) expression from the freshly isolated allantois and chorion and following 24 hours of culture on OP9 cells.

these pieces separately as 24- or 48-hour explants on tissue culture plastic. All three pieces of the allantois gave rise to Runx1⁺ cells (not shown), indicating that Runx1 expression may ultimately initiate throughout the length of the allantois.

To assess whether the chorion also expressed Runx1, we isolated chorions and cultured them as explants for 48 hours on tissue culture plastic (Fig. 3B). Runx1⁺ cells persisted in chorionic explants, and most had crawled off the explant. As we did not separate chorionic mesoderm from ectoderm, we do not know which chorionic component gave rise to the Runx1⁺ cells in the explant culture, but given that chorionic mesoderm is Runx1⁺, and that mesoderm is characteristically migratory, we suspect that the plated Runx1⁺ population was derived from chorionic mesoderm.

In addition to Runx1, we also analyzed the expression of another hematopoietic marker, Sca1, as visualized with a Ly6a/GFP transgene (de Bruijn et al., 2002; Ottersbach and Dzierzak, 2005) in allantoises and chorions before and after explant culture. Ottersbach and Dzierzak (Ottersbach and Dzierzak, 2005) reported that Ly6a/GFP is expressed in the extra-embryonic ectoderm and ectoplacental cone beginning in pre-streak-stage conceptuses. Consistent with their findings, we observed Ly6a/GFP expression in the freshly isolated chorion but not in the allantois (Fig. 3D). After 24 hours of explant culture, both the allantois and the chorion expressed Ly6a/GFP (Fig. 3D).

Thus, on the basis of these observations, we conclude that Runx1, one of the earliest markers of definitive hematopoiesis, is expressed in both the allantois and in the chorionic mesoderm. Furthermore, Runx1 expression in the allantois and the chorion is independent of fusion and is therefore intrinsic to these two tissues.

The allantois and chorion have hematopoietic potential

We next assessed the hematopoietic potential of allantoises and chorions isolated from early headfold to 1-2 s conceptuses, before a circulatory continuum is established between the major vascular systems in the conceptus. We first cultured allantoic and chorionic explants on OP9 stromal cells (Suzuki and Nakano, 2001) in the presence of hematopoietic cytokines that support the growth and differentiation of myeloid lineage cells (IL3, IL6, G-CSF, GM-CSF). Both the allantois and chorion from wild-type conceptuses gave rise to abundant round nonadherent cells (Fig. 4C,E) at efficiencies that were equivalent to those seen with the PAS, an established hematopoietic territory (Table 1). In contrast, allantoises and chorions isolated from Runx1-deficient conceptuses failed to yield

round nonadherent cells (Fig. 4D,F), consistent with the strict requirement for Runx1 in establishing definitive hematopoiesis (Okuda et al., 1996; Wang et al., 1996).

To demonstrate that the nonadherent cells were derived from the conceptus and not a maternal source, we isolated allantoises and chorions from conceptuses derived from crossing a transgenic β -actin/GFP male mouse with a wild-type female mouse. In both allantois and chorion cultures the nonadherent cells were GFP⁺, indicating that they were derived from the conceptus and not from the mother (Fig. 4G,I). Some of the nonadherent GFP⁺ cells also expressed the pan-leukocyte marker CD45, and many appeared to be macrophages based on their ability to uptake DiI-Ac-LDL (Fig. 4H,J). Runx1 is required for the emergence of CD45⁺ cells, including yolk sac macrophages, but it is not required for primitive erythropoiesis (Lacaud et al., 2002; Li et al., 2006; Mukoyama et al., 2000; Okuda et al., 1996; Wang et al., 1996). Cells expressing CD45, c-kit, or both antigens were detected in nonadherent cells collected from both the allantois and chorion explants from wild-type conceptuses (Fig. 4K), while cells expressing these markers were absent in cultures from Runx1-deficient conceptuses. Many of the CD45⁺ cells generated from allantois and chorion explants cultured in SCF, IL3, IL6, Flt3-L, G-CSF and GM-CSF expressed either or both the myeloid cell markers Gr-1 and Mac-1 (Fig. 5A), and cytospin preparations accordingly revealed myeloid lineage cells in the cultures (Fig. 5B).

As a second approach we performed hematopoietic progenitor assays following 5 days of OP9 explant culture in the presence of SCF, Flt-3 ligand and EPO, while omitting G-CSF, GM-CSF, IL6 and IL3 in order to preferentially encourage the proliferation of erythroid progenitors. We found that granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM), granulocyte-macrophage (CFU-GM) and erythroid (BFU-E) progenitors were present in the explant cultures (Fig. 5C). Hematopoietic progenitors were also found in explants following 2 days of culture on plastic in 50% rat serum (Fig. 5C); they appeared at the same time and under the same conditions as the expression of Runx1 in the explants (Fig. 3A,B). We analyzed β -globin gene expression in individual colonies by RT-PCR to determine whether the BFU-E colonies contained primitive or definitive erythroid cells. The erythroid colonies expressed β -major globin but not $\epsilon\gamma$, the latter of which is a specific marker of primitive, yolk-sac-derived erythroid cells (Brotherton et al., 1979; Wong et al., 1983), indicating that the BFU-E colonies consisted of definitive erythroid cells.

DISCUSSION

The chorio-allantoic placenta of eutherian mammals is a composite of two organs: the umbilical cord and the chorionic disk. Two progenitor tissues of the placenta, the allantois and the chorion, are initially physically well separated in the conceptus, and then fuse to form the chorionic labyrinth, where exchange between the fetus and mother takes place. Recent studies have suggested that the murine placenta is an enriched source of hematopoietic progenitors, including CFU-GM, CFU-GEMM, BFU-E and high-proliferation-potential colony-forming cells (Alvarez-Silva et al., 2003). By 11.0 dpc, the placenta is also a source of long-term repopulating hematopoietic stem cells (Gekas et al., 2005; Ottersbach and Dzierzak, 2005). However, whether these cells were derived from other hematopoietic sites within the conceptus, such as the yolk sac and aorta-gonad-mesonephros (AGM) region, or from the allantois and/or chorion themselves was not known.

In this study, we have demonstrated that both the allantois and the chorion have hematopoietic potential. The potential of both tissues was revealed after culturing them as explants on OP9

Table 1. Efficiency at which OP9 explant cultures yielded hematopoietic cells (HC)

Tissue	Stage	Number of explants	% yielding HC*
Allantois	EHF [†]	17	77
Allantois	LHF [‡]	22	90
Allantois	1-2 s	8	100
Chorion	EHF to 1-2 s	27	90
PAS [§]	EHF to 1-2 s	22	96
Embryonic ectoderm [¶]	EHF to 1-2 s	10	10

*Cultures included SCF, Flt3-L, IL3, IL6, G-CSF, GM-CSF and 2-ME, and were scored for the presence of abundant nonadherent cells as seen in Fig. 4C,E.

[†]Early headfold stage.

[‡]Late headfold stage.

[§]PAS, para-aortic splanchnopleura.

[¶]Isolation of the embryonic ectoderm is described in the Materials and methods (see also Fig. 2C).

stromal cells in the presence of hematopoietic cytokines, or by CFU-C assays following a 2 day explant culture on plastic. Explants formed myeloid lineage cells and progenitors for definitive erythroid cells whereas *Runx1*-deficient explants did not. Thus, our results strongly suggest that *Runx1* expression identifies hematopoietic progenitor cells within both the allantois and chorion of the intact conceptus.

Downs and colleagues originally showed that the murine allantois produced very few, if any, erythroid cells when grafted into the exocoelomic cavity or into the allantois of unlabeled host conceptuses (Downs et al., 1998; Downs and Harmann, 1997). However, ectopically transplanted allantoic cells from the distal, middle and proximal portions of the allantois did contribute extensively to the endothelium of the dorsal aorta and the

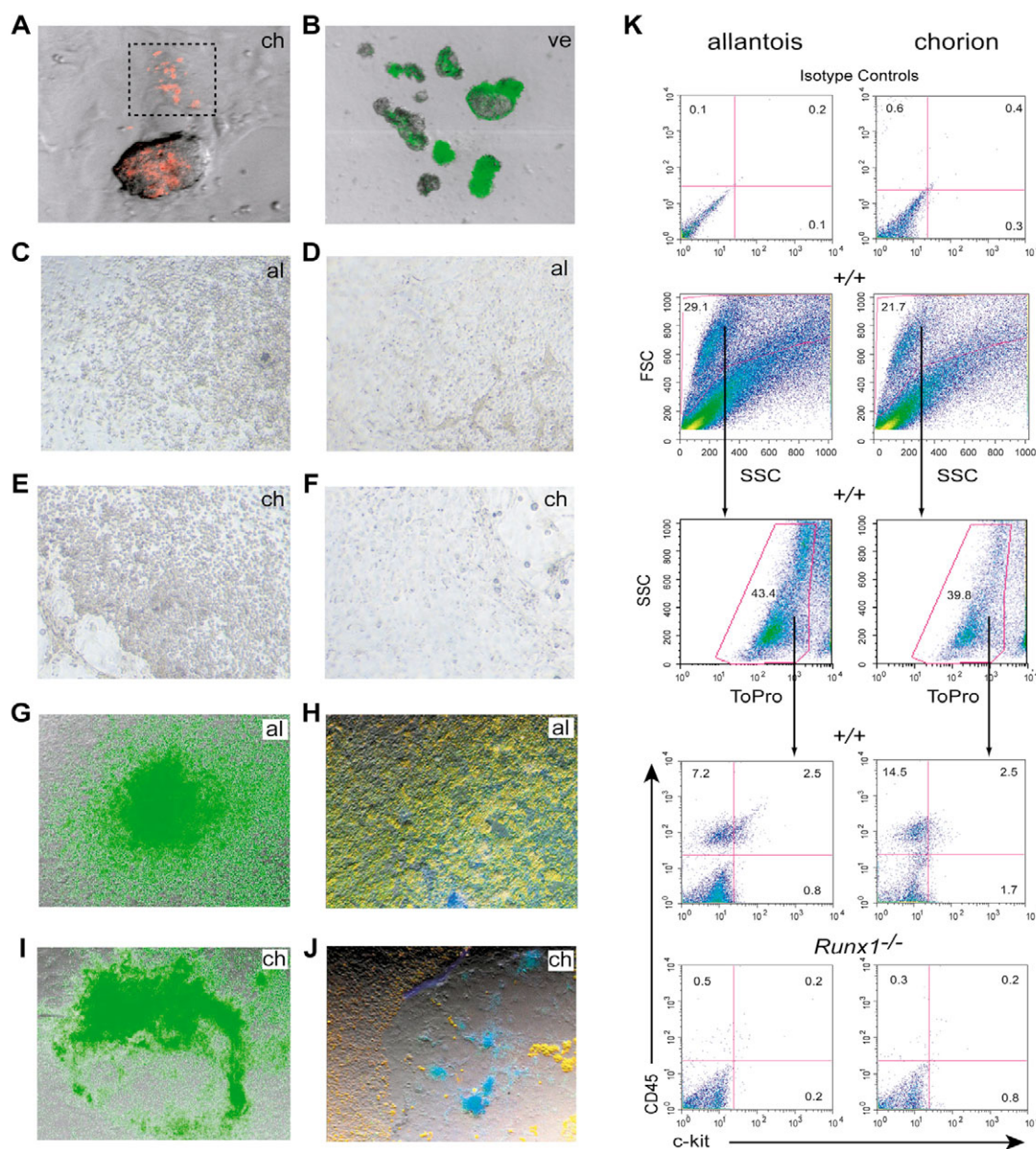


Fig. 4. The pre-fusion allantois and chorion have hematopoietic potential. (A) Explant of ConA 488 (red) labeled chorion following 48 hours of culture on OP9 stromal cells. ConA 488 positive cells that crawled off the explant are boxed. (B) Explant of ConA 594 labeled visceral endoderm (ve) after 48 hours on OP9 cells. (C) Allantois cultured for 7 days on OP9 cells in the presence of SCF, IL3, IL6, Flt3-L, G-CSF, GM-CSF and 2-ME. Note the presence of abundant, nonadherent round cells. (D) Allantois OP9 culture from a *Runx1*^{+/+} conceptus. No nonadherent round cells are present. (E) Chorion OP9 explant culture, same conditions as in panel C. (F) Chorion OP9 culture from a *Runx1*^{-/-} conceptus. (G) Allantois OP9 culture from a β -actin/GFP transgenic conceptus, showing that nonadherent cells originate from the conceptus. (H) Allantois OP9 culture from β -actin/GFP transgenic conceptus, stained with Dil-Ac-LDL (red) and anti-CD45 (blue). GFP⁺ Dil-Ac-LDL⁺ cells are yellow and GFP⁺ CD45⁺ cells are cyan. (I) Chorion OP9 culture from a β -actin/GFP transgenic conceptus. (J) Chorion OP9 culture from a β -actin/GFP transgenic conceptus, labeled with Dil-Ac-LDL and anti-CD45 as in panel H. (K) Representative FACS analysis of three pooled allantois and chorion explants from +/+ and *Runx1*^{-/-} conceptuses cultured on OP9 cells for 14 days in the presence of SCF, IL3, IL6, Flt3-L, G-CSF, GM-CSF and 2-ME and stained for CD45 and c-kit. The explants were cultured separately, and three allantoises or chorions were pooled for FACS analysis in these plots. Cells in each gate were analyzed in the plots below. The experiment was performed three times, with a total of ten +/+ and seven *Runx1*^{-/-} allantoises and chorions.

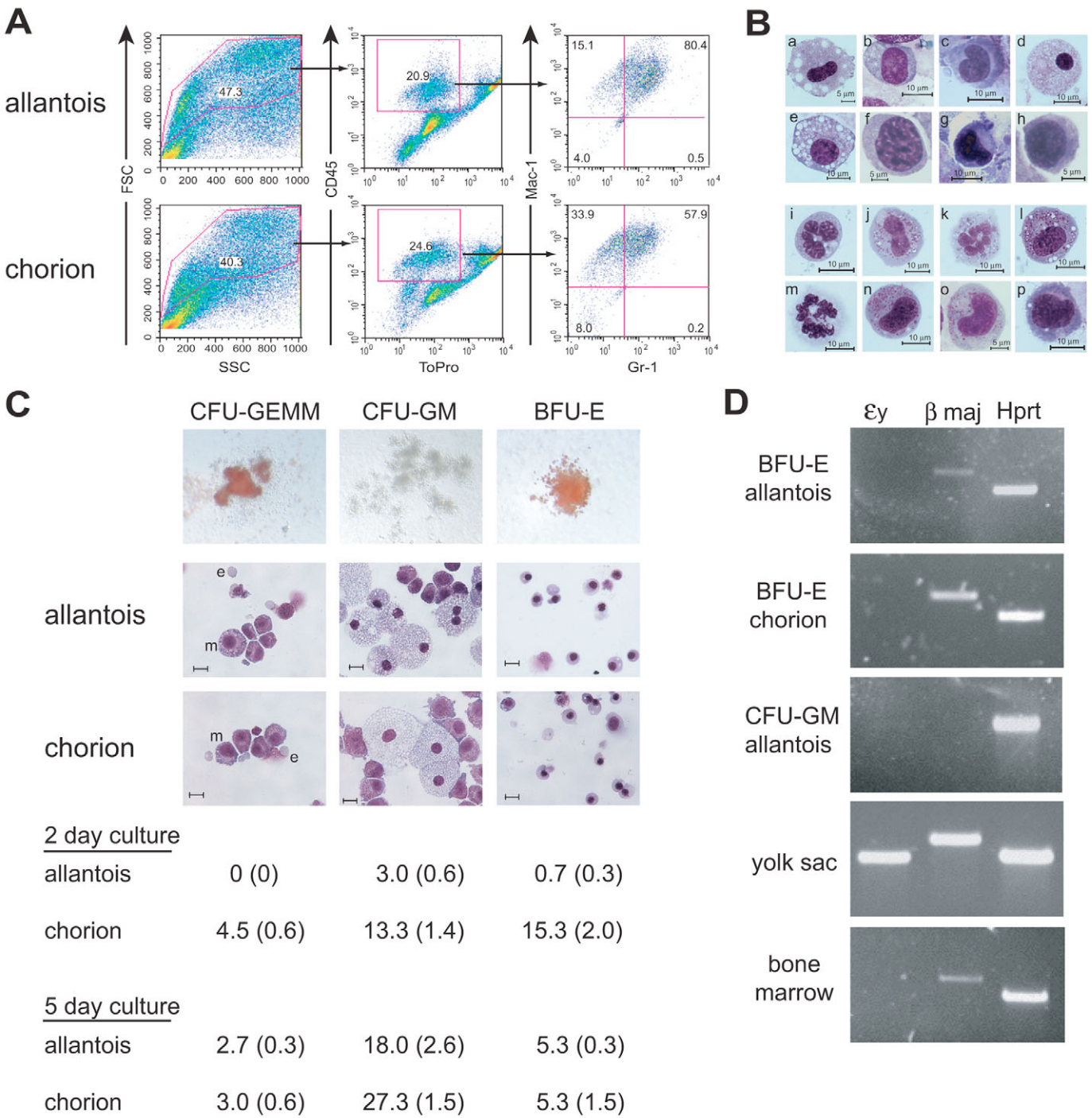


Fig. 5. Myeloid and definitive erythroid potential of allantois and chorion explants. (A) FACS analysis of ten pooled allantois and nine pooled chorion explants following culture for 14 days on OP9 stromal cells in the presence of SCF, IL3, IL6, Flt3-L, G-CSF, GM-CSF and 2-ME. $n_{\text{experiments}}=4$, $n_{\text{allantois}}=32$, $n_{\text{chorion}}=25$. (B) May-Grunwald Giemsa stained nonadherent cells isolated from allantois and chorion explants cultured on OP9 cells under the conditions described in A. a-h, allantois explants; i-p, chorion explant cultures; a-c, i-n, monocytes; d-e, macrophages; f-h, o-p, myelocytes. (C) Methylcellulose colony forming assays from the allantois and chorion. Six conceptus equivalents of allantoises and chorions were cultured together as explants, disaggregated and plated in methylcellulose. The top three panels are representative colonies. (Bottom) May-Grunwald Giemsa-stained cytospin preparations of individual colonies from the allantois and chorion cultures. e, erythrocyte; m, myeloid cell; bar=10 μm . Colony numbers per six conceptus equivalents (\pm s.e.m. in parentheses) following 2 days of explant culture in the presence of 50% rat serum on plastic, or after 5 days of explant culture on OP9 cells in the presence of SCF, Flt3-L and EPO were averaged from three independent experiments. (D) RT-PCR for globin gene expression ($\epsilon\gamma$ and β major). BFU-E or CFU-GM represents individual colonies from either the allantois or chorion methylcellulose cultures. Yolk sac was isolated from ~9.0 dpc conceptuses as a positive control for $\epsilon\gamma$ expression, and bone marrow was used as a negative control.

mesenchyme directly adjacent to it within the PAS of the host embryo (Downs and Harman, 1997). The PAS is an established intra-embryonic hematopoietic territory and, between the headfold and 10 s stages, contains multipotent hematopoietic activity that could be revealed following explant culture on stromal cells (Cumano et al., 1996; Cumano et al., 2001). In retrospect, the extensive contribution of the allantois to the dorsal aorta and periaortic mesenchyme upon transplantation (Downs and Harman, 1997) is consistent with the hematopoietic potential demonstrated in vitro in the current study. Importantly, the hematopoietic potential of the allantois was demonstrated here in all three allantoic subregions of early headfold stage conceptuses, before vascular continuity between the allantois and yolk sac was achieved. Therefore the hematopoietic progenitors must have arisen de novo from the allantois and not via the circulation from the yolk sac or the embryo proper.

Although we did not separate the chorionic mesoderm from ectoderm, we suspect, based on our results of β -galactosidase staining, that the Runx1⁺ mesodermal component is the source of the hematopoietic cells supplied to the chorion. The site within the allantois that gives rise to hematopoietic cells is not clear; however, as Runx1⁺ cells were located on the outer mesothelial surface, which mediates chorio-allantoic union, it is likely that the allantoic mesothelium is involved, with Runx1⁺ endothelial cells within the base of the allantois being a second potential source.

Very little is known about either allantoic mesothelium or chorionic mesoderm other than that they mediate chorio-allantoic fusion (Downs and Gardner, 1995) via VCAM1 on the allantoic mesothelium (Gurtner et al., 1995; Kwee et al., 1995) and its counter receptor α 4 integrin on chorionic mesoderm (Yang et al., 1995). Once the allantois fuses with the chorion, it is not known whether the fusing surfaces of the allantois and chorion break down, persist or differentiate into other cell types (Downs, 2002). Based on findings presented here, we suggest that cells from one or both of these fusing surfaces persist. In this way, Runx1⁺ hematopoietic cells derived from allantoic mesothelium and/or chorionic mesoderm may then enter the allantoic vasculature during or after its penetration into the chorionic plate. One interesting observation is that Runx1 expression in the chorionic mesoderm and at the site of intersection between the allantois and the chorion very closely resembles that of α 4 integrin, both temporally and spatially (Downs, 2002). Both α 4 integrin and Runx1 are expressed on all long-term repopulating hematopoietic stem cells in the embryo (Gribi et al., 2006; North et al., 2002), lending support to the notion that the Runx1⁺ and α 4 integrin⁺ cells at the chorio-allantoic fusion junction ultimately contribute to hematopoiesis in the placenta.

One of the issues raised by these studies concerns the location of the source of the signal (or signals) that induces the hematopoietic program in the allantois and chorion. Studies in both avian and murine conceptuses clearly show that the induction of the hematopoietic program in mesoderm requires a signal from endoderm. For example, in the mammalian yolk sac, visceral endoderm provides soluble signals that are required for the formation of blood islands (Belaousoff et al., 1998). In the avian embryo, a brief exposure to endoderm can re-specify axial and somatopleural mesoderm, which has no hematopoietic potential, to become splanchnopleural mesoderm that could contribute to the formation of hematopoietic cells in the dorsal aorta (Pardanaud and Dieterlen-Lièvre, 1999). The endodermal signal in the avian allantois was thought to originate from the core of endoderm within the allantois proper. The allantois of avian embryos emanates from the intra-embryonic splanchnopleural mesoderm and contains

within it endoderm derived from the primitive hindgut (Caprioli et al., 1998). However, whereas the allantois of many eutherian mammals exhibits an endodermal component (Mossman, 1937), the allantois of the house mouse is thought to consist entirely of extra-embryonic mesoderm that contains within it a core of Brachyury-positive cells and is surrounded by a mesoderm-derived mesothelial layer (Duval, 1891; Inman and Downs, 2006; Mossman, 1937). A re-examination of the murine allantois for an endodermal component using modern techniques and markers may be warranted.

The distal tip of the allantois is the 'oldest' allantoic tissue, having emerged first from the primitive streak (Downs et al., 2004; Kinder et al., 1999; Lawson et al., 1991), whereas the cluster of cells within the base of the allantois emerges later (Kinder et al., 1999). We hypothesize that signals separated either temporally or spatially may activate Runx1 expression in the distal portion of the allantois and within the base. It is tempting to speculate that, as the mesoderm at these two sites originates within the primitive streak over a prolonged period (6.75-8.5 dpc) (Beddington, 1982; Copp et al., 1986; Downs, 2002; Kinder et al., 1999; Lawson et al., 1991; Tam and Beddington, 1987), these signals may be encountered at different times in the primitive streak. However, as so little is known about the role of the streak in specification of allantoic mesoderm (Downs et al., 2004), further experimentation is needed to address this notion. Moreover, as little is known about chorionic mesoderm and ectoderm, the signals responsible for Runx1 expression in the chorionic mesoderm are completely unclear at this time.

In summary, our findings strongly suggest that the placenta itself is a hematopoietic organ. To what extent hematopoietic activity emerges de novo from the placenta, or comes from other sites of hematopoietic emergence awaits further investigation.

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